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Activity of Iron oxide nanoparticles on bacterial biofilm formation

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Abstract

Biofilm is important virulence factor play an essential role in bacterial pathogenicity through trigger antimicrobial agents. Nanoparticles have antimicrobial properties and the antimicrobial activity depend on nanoparticles size and concentration. This study demonstrated the antimicrobial activity of iron oxide nanoparticle against Gram-positive and Gram-negative bacteria within 20 hours. Particles of iron oxide was synthesized via co-precipitation method. The particles obtained had an average diameter 85.9 nm; the particles were used to inhibit *Staphylococcus aureus*and *Escherichia coli* biofilm on polystyrene surface. The bacteria were added in 96-well plates to incubate with iron oxide nanoparticles and without iron oxide nanoparticles as control. The biofilm was measured using the safraninstaining method that showed variable results depending on bacterial species. However, It was seen that exposure of cells to iron oxide particles showed increased biofilm formation on polystyrene plates.The highest augmentation was recorded for *S. aureus*in a concentration of 0.5mg/ml and approaching (78.8%) highly significant $p \leq 0.05$ and the augmentation was not achieved in a concentration of 50 mg/ml $p>0.05$, The augmentation of *S. aureus*in a concentration of 5mg/ml gave (75.8%).Augmentation percentage of *E.coli* at 50 mg/ml was 36.9% not significant $p>0.05$ compared with the control, while at concentration 5mg/ml showed (59.8%) augmentation $p>0.05$. Percentage augmentation of *E.coli* in concentration of 0.5mg/ml gave (43.4%) non- significant$p>0.05$.

Sera proteins of patients infected with*S. aureus*and *E. coli* were exhibited reactivity to*S. aureus*and *E. coli* antigens (whole cells) as: three strains of *S. aureus*antigens agglutinated in titer 1:128 of patients sera (antibodies) while the remaining werethree *S. aureus*antigens agglutinated with the patients sera in titer 1:16, 1:64 and 1:256 respectively. *E. coli*antigens (whole cells) reactivitywith patients serashowed: two of antigens agglutinated in titer 1:32 of patients sera (antibodies) while three of *E. coli* antigens agglutinated in titer 1:16 , 1:64 , 1:128 and 1:256 respectively.

Sera proteins of patients infected by *S. aureus& E. coli* exhibited reactivity with *S. aureus&E. coli* antigens (whole cells) as The highest agglutinated in titer 1:256, 1:128 respectively of patients sera (antibodies). Whereas results lowest agglutinated in titer 1:16, 1:8was appeared on the isolates *S. aureus& E. coli*.

Our result indicated that iron oxide nanoparticles have not antibiofilm activity against bacteria cells within 20 hours for *E.coli* and *S aureus* except at concentration 50mg/ml for *S.aureus* gave (18.2%) inhibition rate. The IONPs efficacy depends on the incubation time, bacterial strain and nanoparticles concentration. In addition anti*S aureus*antibodies titer more than anti*E.coli*antibodies, So the biofilm of gram positive bacteriacan be control by IONPs more than gram negative bacteria biofilm in patients.

Keywords:*Escherichia coli, Staphylococcusaureus,* Iron oxide nanoparticles,Biofilm, Antibodies, Antigens.

INTRODUCTION

Bacteria are ubiquitous in environment, 99% of bacteria in nature can form biofilm (1).Most of these bacteria are opportunistic pathogen and associated with chronic infection (2).Bacteria in biofilm can persist in varies environment and resistance to antibiotics(3).Antibiotic resistance can develop in biofilms due to antibiotics cannot penetrate biofilm layersinadditionmetabolicand physiological activity ofbacteria are different between biofilm layers(4).Recently attention towards develop a new antibiofilmtherapies such as metal nanoparticles. Metal nanoparticleshavelarge surface area, small size which can attach,interactand penetrate the biofilm layers. These particles then damage the cell membranes and DNA leading to cell death (5). Iron oxide nanoparticles (IONPs) have magnetic properties, low toxicity and biocompatible compared with other nanoparticle system thereforeexpanding use of IONPsin biomedical research. IONP types increased due to the variation in preparation methods.Superparamagnetic iron oxide nanoparticles are composed of iron oxide particles size between 50 and 180 nm.Ultra small superparamagnetic iron oxide nanoparticles are nanoparticles size between 10 and 50 nm nanoparticles (USPIONs) and very small SPIONs which are smaller than 10nm in diameter (6). The use of nanoparticles in biomedical application depend on nanoparticles diameter.The most pathogens associated with biofilm formation in environments: *E. coli O157:H7, L. monocytogenes, Staphylococcus aureus* and *Staphylococcus epidermidis*, and non-pathogenic *E. coli K12* (7). So in our recent studies, we showed effect of iron oxide in nano size on biofilm formation for *E. coli* and *S. aureus*isolated from nosocomail infections.

EXPERMENTAL

Bacterial strains

Staphylococcus aureus, and *Escherichia coli*were used for this study. Bacterial strains used in this study were obtained from the nosocomial infected patients. Bacteria were first grown aerobically overnight at 37°C in tryptone soy broth (TSB; Hi media, Mumbai, India) for 20 h.

Iron oxide nanoparticles preparation

Iron oxide nanoparticles (IONP) were prepared using Kedar *etal.*, method (8). The black precipitate was indicated IONPs formation.

Characterization of iron oxide nanoparticles

Nanoparticles morphology determined by Scanning probe microscope (SPM) analysis in the department of chemistry, Faculty of Science, University of Baghdad. Baghdad–Iraq.

Fourier transform infrared spectroscopy (FTIR) of nanoparticles was done at the department of chemistry, Faculty of science, university of AL-Mustansiryiah. Baghdad–Iraq.
All samples were scanned over a range of 600–4000 cm^{-1} on a 2400S spectrophotometer.

Effect of Iron-Oxide Nanoparticles on Biofilm Growth
In this study, bacterial adhesion on polystyrene well plate and bacterial with IONPs adhesion on polystyrene well plate were compared. 20 µl of *Staphylococcus aureus* and *Escherichia coli* culture suspensionwas added to each well. Then, 180 µl of the iron-oxide nanoparticles were introduced in different concentrations (50 mg/ml , 5mg/ml and 0.5 mg/ml). Thereafter, biofilms were allowed to grow for 20 h. Subsequently, wells were washed with sterile water to remove unbound bacteria then, 200 µL of 0.1% safraninstaining was added to each well. Plates were incubated for 10 min. The wells were washed with sterile water and allowed to dry completely. Biofilm development was assessed by measuring the optical density (absorbance at 490 nm) using a BioTic ELISA reader (ELx800)(9). Experiments were performed in triplicate with separately cultured bacteria.

Bacterial Antigen Preparation (Whole Cell Killed by Heat)
For agglutination tests, cells were prepared as described by (10).

Antibodies Production
Blood samples were drawn from patients, serum was obtained after the blood was clotted for at least30min, then centrifuged for 15 min at 1500 rpm (11). Serums werekeptfrozen at (−20°C) tillwere required.

Agglutination Antigens with Antibodies
1- All wells were Coating via 50 µl (1.5 × 10 ^8 CFU/mL) of*S. aureus*& *E. coli*antigen.
2- Serial dilutionsof patient's serum antibodies were added(1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256) to all wells.
3- Distinguished the macroscopically agglutination titers, also evaluated microscopically agglutination (12).

Statistical analysis
Experiments were performed in triplicate. Data are represented as a mean with standard deviation. For statistical analysis ANOVA was performed by a computer program for Epidemiological statistics and a p value <0.05 was considered to be significant.

RESULTS:

A. Characterization of iron oxide nanoparticles
Successful iron oxide nanoparticles formation was confirmed by FTIR fig. 1. The appearance of characteristic bands such as the presence of Fe-O bond (>850 cm^{-1}) is evident. It appears that the absorption frequencies at low wave numbers between 850 and 400 cm^{-1} came from vibrations of Fe–O bonds of iron oxide (13).
Particle sizes determined by SPM indicated that the nanoparticles are close to spherical and monodispersed. The particle size distribution and histogram seen in fig. 3.has an average diameters of 85.9 nm and ranging from (70- 105) nm fig. 2.

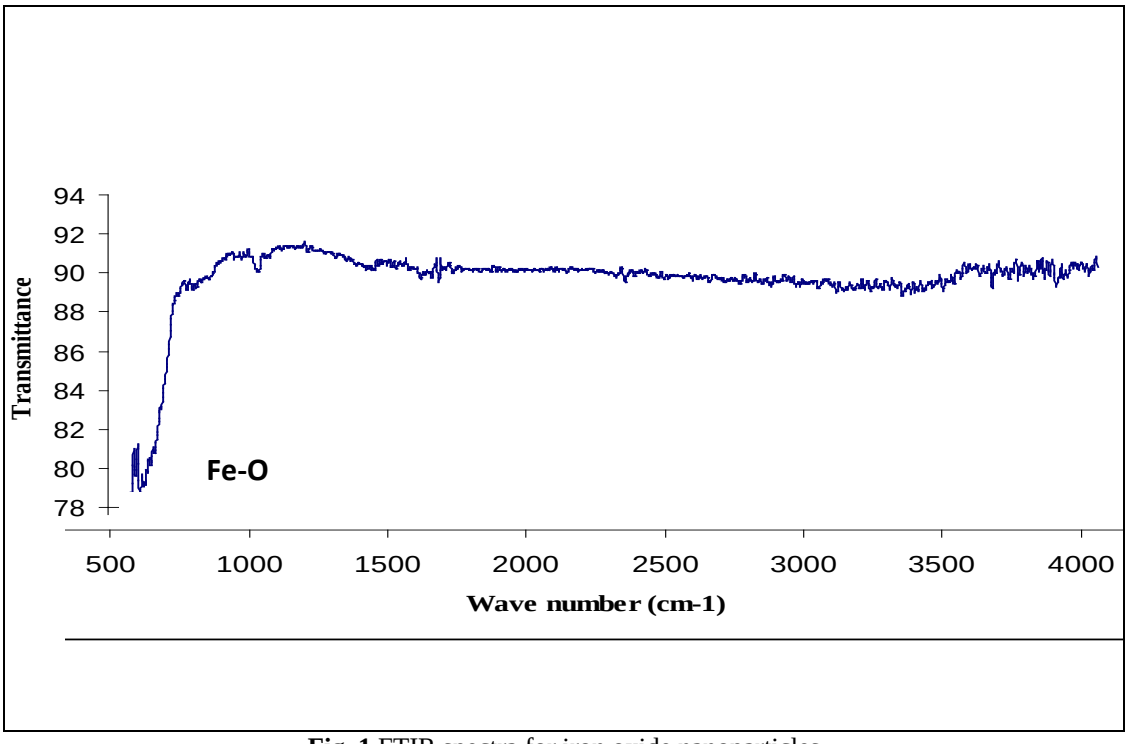

Fig. 1.FTIR spectra for iron oxide nanoparticles

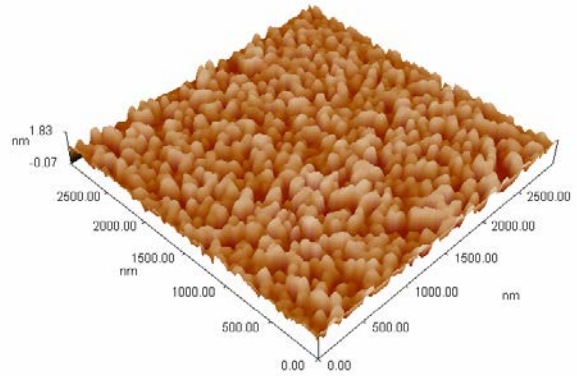

Fig. 2.A; SPM iron oxide nanoparticle

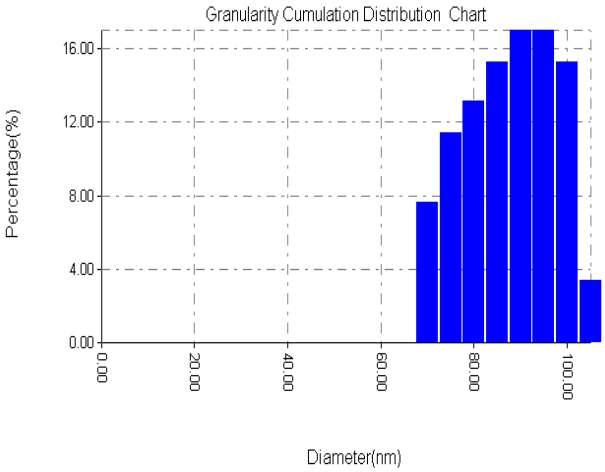

Fig. 3.SPM particle size distribution histogram of iron oxide nanoparticle.

B. Effect of Iron oxide nanoparticles on E. coli biofilm formation.

In table (I), (II) Iron oxide nanoparticle was used at concentration of 50, 5, 0.5 mg/ml on*E.coli* biofilm. The results showed inductionin the amount of biofilm biomass in the 20h treatment time compared with control.

TABLE I Effect of concentration of iron oxide nanoparticles on *E.coli*biofilm formation

Concentration mg/ml	% Augmentation (Mean)
50	36.9%
5	59.8%
0.5	43.4%

Augmentation was calculated using the formula :
% augmentation= Test O.D -Control O.D×100
Control O.D

TABLE II Optical density (OD) biofilm of *E.coli* with iron oxide nanoparticles on polystyrene surface

Concentration mg/ml	OD at 490nm (Mean ± SD)	P-Value	T-test	Standard error
50	0.167 ± 0.032	0.08**	2.2	0.02
5	0.195 ± 0.06	0.11*	2.05	0.036
0.5	0.175 ± 0.015	0.01***	4.4	0.012
Control	0.122 ± 0.014			

*non significant
**quite non significant
* significant
control/ is biofilm of bacteria without nanoparticles

C. Effect of Iron oxide nanoparticles on S.aureus biofilm formation.

Iron oxide nanoparticle was used at concentration of 50, 5, 0.5 mg/ml on*S. aureus* biofilm in table (III), (IV). The results indicated ainduction in the amount of biofilm biomass in the 20h treatment time compared with control.

TABLE III Effect of concentration of iron oxide nanoparticles on *S. aureus*biofilm formation

Concentration mg/ml	% Augmentation (Mean)
50	0%
5	75.8%
0.5	78.8%

augmentation was calculated using the formula :
% augmentation=Test O.D -Control O.D×100
ControlO.D

TABLE IV Optical density (OD) biofilm of *S. aureus*with iron oxide nanoparticles on polystyrene surface

Concentration mg/ml	OD at 490nm (Mean ± SD)	P-Value	T-test	Standard error
50	0.27 ± 0.05	0.179*	1.62	0.037
5	0.58 ± 0.34	0.27*	1.26	0.198
0.5	0.59 ± 0.08	0.007***	5.034	0.052
Control	0.33 ± 0.04			

*Non -significant
* verysignificant
control/ is biofilm of bacteria without nanoparticles

Antigen - Antibodies titration

Results in table V showed sera from patients reactivity with *S. aureus& E. coli* antigens (whole cells) as three *S. aureus*antigens agglutinated in titer 1:128 of patients sera (antibodies) while 4 , 2 ,5 of *S. aureus*antigens agglutinated in the patients sera in titer 1:16, 1:64 and 1:256 respectively. While, results in *E. coli* showed sera from patients reactivity with *E. coli* antigens (whole cells) as: two of antigens agglutinated in titer 1:32 of patients sera (antibodies) while 1, 3 , 4 ,6 of *E. coli* antigens agglutinated in the patients sera in titer 1:8 ,1:16, 1:64 and 1:128 respectively.

TABLE V Agglutination of antigens (whole cells of *S. aureus& E. coli*) against antibodies in sera of patients

Concentration ofcells of *S. aureus&E. coli* antigens(whole cells)	No.of bacteria	Titer of antibodies(*S. aureus*)	Titer of antibodies*(E. coli)*	control (serum)
(1.5×10^8 CFU/mL)	1	128	8	0
	2	64	32	
	3	128	16	
	4	16	64	
	5	256	32	
	6	128	128	

DISCUSSION:

Increased biofilm formation were seen for both bacteria (table I, II, III and IV). The highest augmentation significantly was seen at concentration 0.5mg/ml IONPs gave 78.8 % for *S.aureus.* While the highest augmentation for *E. coli* was seen at concentration 5mg/ml gave 59.8 %.This results indicated that *S.aureus* biofilm was more induced by IONPs than *E.coli* biofilm in consequence of*S aureus* biofilm (0.33 ± 0.04) was stronger than *E.coli* biofilm (0.12 ± 0.01), therefore IONPs used in bacteria biofilm building at low concentration through 20 hours this results was compatible with (14) who's mentioned that increased biofilm mass dependent on IONPs size .Iron used in biofilm regulation (15), (16), (17) and (18). In concentration of 50 mg/ml for *S.aureus* shown non significant decreased in biofilm formation and the inhibition gave 18.2%. No inhibition seen in *E.coli*but at concentration 50 mg/ml gave the lowest augmentation rate (36.9%) consequently increased IONPs concentration decreased biofilm formation (19), (20), (21). In addition *S.aureus* as gram positive cell wall different from *E.coli* gram negative cell wall.In gram negative bacteria,the cell wall is protected by outer membrane . While gram positive bacteria contain different types of peptidoglycan,vary in the amino acid (22), So we seen different results depending on the structure of the cell walland the mechanisms to inter the IONPs.

Taylor and Webster showed that iron-oxide nanoparticles in a concentrationrange of 0.01 to 2mg/mL were inhibited 25% of *S.epidermidis biofilm* at 48 h (23). And, similar results were observed by (24) on *S. aureus*biofilms at 24 hours. In contrast, (25) showed an increase in *P. aeruginosa* biofilm biomass in the presence of 0.2mg within 16 hours. (25) results was similar with current studieswith iron oxide nanoparticles on *S. aureus and E.coli*biofilmswithin 20 hours, consequently efficacy of IONPs to kill bacteria biofilm depends on the incubation time and nanoparticles concentration. So when the concentration of the nanoparticles increase the time is decrease to inhibited the biofilm and versa visa.

Agglutination are based concerning the availability over antibodies within patient sera that can react with specific antigens or structure visible clumps, but formation on biofilm may shield bacteria out of the assignment regarding antibodies (26).

The positive reaction between surface antigens about microorganism and the antibodies, as reflect on consideration on as like a good method ancient for diagnose infection then become awake of bacterial isolates with the aid of discovery on bacterial-specific antibodies of samples.

Study showed the polyclonal antibodies go commotion with hateful non-specific antigens, the awareness about antibodies who was once ancient toughness no longer ample because inhibition about the function about partial proteins (27). Specific antibodies blocked biofilm development at the initial attachment and aggregate stages, deletion and inhibited normal biofilm formation. So particular antibodies additionally respect namely opsonins after enhance neutrophil binding, motility, and biofilm engulfment.Vaccination against, or treatment by antibodies reactive to proteins may deliver targets for usage against a extensive spectrum of gram-positive bacteria (28).

Commonly*S. aureus& E. coli* have aninformed capacity to link non- specifically to bare polymer surface, this combination can be blocked by cover the surface with altered proteins, vaccine or as a goal in passive immunotherapy or prophylaxis. (29).Consequently, some of antigenicbacterial proteins are forthcoming targets for immunotherapy, which accomplish available a novel strategy for control ofbacteriacontamination and could possiblydiminution infection and have aimportant effect on human health.

Antibodies can inhibiting biofilm formation without killing the bacteria (30), (31), in addition most important pathogenic bacteria have capsular carbohydrate , LPS and S layer that protect the bacteria against complement lysis, antibodies and phagocytosis (32), (33). And we are mention that IONPs can kill biofilm bacteria , therefore we can used antibacterial antibodies conjugated with IONPs to kill any biofilm in vivo or in vitro.

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